EXHIBIT B

Lenti-MEF2CA-hESC NPC-Patent

Figure legends

Fig.1. Biochemical characterization of lenti-MEF2CA. (A) The components of lentiviral expression system. Diagram shows the lentiviral transfer vector harboring PGK promoter-MEF2CA-IRES-GFP. pCMVΔR8.74 encodes the essential viral genes (GAG, POL, TAT, and REV) for virus production and integration. pMD.G plasmid encodes the gene for the coat proteins of Vescicular somatitis Virus-G (VSV-G). (B) Each lentivirus titer was shown based on FACS analysis after infecting in HEK293T cells. (C) The transcriptional activity of lenti-MEF2CA was measured using MEF2 RE-MHC-luciferase and (D) the GFP expression level was monitored by Western blot after infecting in SH-SY5Y cells. MEF2CA, constitutively active MEF2C; IG, IRES-GFP; MEF2 RE, MEF2 response element.

Fig.2. Effect of lenti-MEF2CA on neurogenesis in human fetal brain-derived neuroprogenitor cells. (A) Schematic diagram showing the differentiation procedure and lentivirus infection. hFBNP, human fetal brain derived neuroprogenitor cells; PO, poly-L-ornithine; LN, laminin. (B) Representative pictures of lentivirus- infected and migrated cells. Neurospheres were infected with each lentiviruses and double immunostained with an anti-GFP antibody (green) for viral infected cells and an anti-TuJ1 (red) for newly generated neurons, or an anti-GFAP (red) for neural precursor cells. Quantification of the data was shown by histogram. TuJ1+ or GFAP+ cells were counted over GFP+ population (up), and over total cells (down). Values are mean S.E.M. (n=10; *, ** $p<0.001$; ***, ♦♦ $p<0.01$, Bonferroni's multiple comparison test). Scale bar, 25 μm. (C) The longest neuronal process per cell was measured by Neuron J software. Values are mean S.E.M (n=50 per each lentivirus infection; **** $p<0.001$, Bonferroni's multiple comparison test).

Fig.3. Scheme for the neural differentiation from human embryonic stem cells. The phase-contrast images were taken during differentiation and represent cellular morphology of each developmental step. NIM, neural induction medium; NPM, neural proliferation medium; TDM, terminal differentiation medium; hESC, human embryonic stem cells; hNEP, human neuro epithelial cells; hNPC, human neural precursor cells.

Fig.4. Marker protein expression during differentiation from hESC to neural cells. Cells from D0 (hESC), D6 (hNEP), D40 (2 weeks after 2^{nd} plating) were fixed and stained with stage-specific antibodies. Scale bar, 25 μm

Fig.5. Enrichment of neuronal population in lenti-MEF2CA infected cells. (A) Scheme for the infection and analysis. (B) The representative picture from each lentivirus infected cells. Cells were fixed and stained with anti-GFP antibody for infected cells and with anti-Doublecortin antibody for neurons. Arrows are representing MEF2CA-induced neurons. Scale bar, 25 μm. (C) Quantification of counting results was shown by fold in histogram. Counting was done in four individual experiments. In each experiment, approximately 300 of GFP positive cells were counted.

Fig.6. Electrophysiological activity of MEF2CA-derived neurons. Lenti-MEF2CA-infected rosettes were subjected to sorting based on GFP expression and GFP+ cells were plated on glass cover slips coated with PO/LN and allow to be differentiated for 5 weeks. (A) Represent whole-cell recordings of sodium currents evoked by 100 ms depolarizing steps from -60 to +80 mV in 20 mV increments following a 300 ms prepulse to -90 mV (up). Current/voltage (*I*/V) relationship of peak amplitude I_{Na} currents reveals a TTX-sensitive component representing I_{Na} current (down). (B) GABA-evoked currents were recorded under voltage clamp in the whole-cell configuration (up). GABA-evoked responses exhibited a reversal potential around -51 mV (down).

Methods

Lentivirus generation and characterization

At day 0, HEK293T cells were plated in 6 of 150-mm dishes with 15 x 10^6 cells/ml. At day 1, cells were almost 80% confluent. For transfection, three plasmids (lentiviral transfer vector, pCMVΔR8.74, and pMD.G) were mixed in a ration of 3:2:1 (30, 20, 10 μg each) and made up to 1350 μl with H_20. 150 μl of 2.5 M of CaCl2 were added by dropwise. Finally 1500 μl of 2x HBSS solution [8 mg NaCl, 0.37 mg KCl, 0.19 mg Na2HPO4.7H20, 1 mg Dextrose, 5 mg Hepes, pH 7.2 in 500 ml] was added dropwise with constant vortexing. The mixture was incubated for 15 min at room temperature and applied to the cells in 16 ml medium. Cells were incubated for 7 hr in a 5% CO2 incubator at 37 °C and then replaced with viral harvesting medium [Ultraculture medium, Bio-whittaker] and further incubated for 48 hrs. at day 3, culture medium was collected and centrifuged for 10 min at 400 g, and then filtrated through 0.45 μm filter (low protein binding-cellulose acetate). To concentrate virus, the supernatant was centrifuged in swing-bucket SW32 (Beckman) at 20,000 rpm for 2.5 hrs at 4 °C. Viral pellet was re-suspended in 100-150 μl of remaining medium for 3 hrs at 4 °C. The concentrated virus was stored at -80 °C after aliquot. To do titration, HEK293T cells were plated in 12-well plate with 2 x 10^5 cells/ml one day before infection. Cells were infected with different virus volume (0.1, 0.3, 1.0, and 3.0 μl) at following day and incubated for 6-7 hrs. After washing twice with 1x PBS, cells were further incubated under fresh growth medium for 3 days. Cells were trypinized and re-suspended in 2% FBS/1xPBS and analyzed by FACSsort (BD) to calculate GFP positive cells. Virus transfection unit (TU/ml) was calculated by a formula {[(%GFP/100) x cell number at day of infection]/virus volume (μl)}. To measure the MEF2CA activity, SH-SY5Y cells were plated in 24-well plate with 1x10^5 cells/ml. Next day, each virus was added with MOI 0.5 for 7 hrs, followed by washing once with 1x PBS and cells were cultured for another day. Cells were subjected to transfection with 0.5 μg of MEF2 response element (RE)-MHC-Luciferase plasmid as well as 0.05 μg of Renilla as an internal control per well by Fugene HD (Roche). Two days later, cells were washed and lyzed by 1x Passive lysis buffer (Promega) and then

assayed by Dual-Luciferase reporter assay system (Promega). To monitor the expression of GFP after infection, SH-SY5Y cells infected with MOI 0.5 or 1 were lysed with 1x SDS buffer without reducing agent and bromphenol blue and quantified by BCA (Pierce). 50 μg of total protein was loaded in 4-12% Bis-Tri gel (Invitrogen) and transferred to nitrocellulose membrane. Rabbit anti-GFP antibody (Invitrogen) was used for probing GFP expression.

Lentivirus infection and differentiation of hFBNP

hFBNP were dissociated by accutase (Chemicon) and prepared single cells were infected with pRRL.PGK.GFP.SIN18WPRE (control, 1 x 10^9 IU/ml) or pRRL.PGK.MEF2CA. IRES.GFP.SIN18WPRE (MEF2CA, 1 x 10^8 IU/ml) for 6 h. Infected cells were washed with 1x PBS twice and placed under proliferation medium [Neurobasal medium/2% B27 supplemented with bFGF (20 ng/ml, Sigma) and EGF (20 ng/ml, R&D systmes)] for 5 days to form neurospheres and then plated on coverslips coated with poly-L-ornithine (PO, 50 μg/ml, Sigma)/laminin (LN, 5 μg/ml, Invitrogen) under differentiation medium [bFGF/EGF-free neurobasal/2% B27 medium] for 3 days for neuronal marker expression. Or infected cells were directly plated on PO/LN-coated coverslips and incubated for one day under proliferation medium and then cultured for 7 days in differentiation medium to measure the neuronal length. The longest neurite was picked and the length was measured by NeuronJ (an ImageJ plugin).

hESC culture and neural induction

Undifferentiated H9 of passage 48-69 were cultured on γ-irradiated human foreskin fibroblast (Hs27, ATCC) feeder layer/0.5% gelatin under growth medium [DMEM/F12, 20% Knockout serum replacement, 1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol per 500 ml, all reagents from Invitrogen] supplemented 8 ng/ml bFGF (Sigma). Cells were mechanically subcultured once a week and medium was changed everyday. For neural induction, hESC were incubated under neural induction medium (NIM) [DMEM/F12:Neurobasal (1:1), 2% B27, 1% N2, all from Invitrogen] for 24 h and then dissociated to small clumps by mechanical scrapping. Small clumps were settled down and placed bacterial petri-dish in NIM for another 3 days. Spheres were collected

and moved to a new dish in neural proliferation medium (NPM) [DMEM/F12:Neurobasal (1:1), 1% B27, 0.5% N2, 20 ng/ml bFGF, 20 ng/ml EGF (R&D), 2.5 μg/ml insulin (Sigma)] for 3 days. Spheres were harvested again and placed on laminin (10 μg/ml)-coated dish in NPM for another 4 days to form the rosettes. Under dissecting scope (Leica), rosettes were isolated by a $27G1^{1/4}$ needle and put into ultra low attachment plate (6-well plate, Costar) under NPM. For the infection of hESC-derived rosettes, isolated rosettes were settled down and divided into 3 of 5-ml polystyrene round bottom tubes (BD falcon). Each virus (control, MEF2CA, and Bcl.Xl) was added with MOI 2 and incubated overnight. Infected rosettes were washed with 1x PBS twice and put into ultra low attachment plate under NPM for 4-6 days. After that, rosettes spheres were pass through a $27G1^{1/4}$ needle once and plated on PO(10)/LN(1 μg/ml)-coated plates in NPM (1^{st} plating). One week later, cells were dissociated with accutase (Chemicon) and placed on PO(100)/LN(10 μg/ml) under terminal differentiation medium (TDM) [DMEM/F12:Neurobasal (3:1), 1% B27] (2^{nd} plating). Cells were kept for another 2 weeks to examine the expression of neural markes or dissociated with accutase one week later again for 3^{rd} plating. To examine the effects of each lentivirus infection, cells were fixed at early stage of 2^{nd} or of 3^{rd} plating and stained with rabbit anti-GFP and guinea pig anti-doublecortin antibodies.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 min at RT and permeabilized with 0.25% of Triton X-100 in PBS for 10 min. Each primary antibody information is followed: GFP (rabbit, 1 to 1000, Invitrogen), TuJ1 (mouse, 1 to 1000, Covance), MAP2 (mouse, 1 to 1000, Sigma), GFAP (mouse, 1 to 1000, Sigma), OCT4 (rabbit, 1 to 1000, Santa Cruz), vimentin (mouse, 1 to 1000, Sigma), Pax6 (mouse, 1 to 1000, DSHB), Sox2 (mouse, 1 to 1000, Chemicon), Musashi1 (Msi1, rabbit, 1 to 1000, Chemicon), doublecortin (DCX, guinea pig, 1 to 1000, Chemicon), S100 (Sl00β, rabbit, 1 to 10000, Swant), NeuN (mouse, 1 to 500, Chemicon), synaptophysin (Syn, rabbit, 1 to 1000, Dako), PSD95 (mouse, 1 to 500, Upstate), engrail 1 (Engl, mouse, 1 to 100, DSHB), tyrosine hydroxylase (TH, mouse, 1 to 1000, Pel-freze; rabbit, 1 to 1000, Chemicon). Primary antibodies were applied for 1 h at room temperature and washed with 1x PBS,

three times. Alexa 488 goat anti-mouse or rabbit IgG(1 to 1000, Invitrogen), Alexa 555 goat anti-mouse or rabbit IgG (1 to 1000, Invitrogen), or Alexa 647 goat anti mouse or rabbit IgG (1 to 1000, Invitrogen) were used as secondary antibodies. Rhodamine Red conjugated Stratavidin (Pierce) were used for Biotin labeled anti-guinea pig IgG. Deconvolution microscopy was performed to take the images; Filters for DAPI, CY3, CY5, and FITC were used for three or four-color image capture and a 'nearest neighbor' or 'no neighbor' algorithm for deconvolution (Slidebook software, Intelligent Imaging Innovations).

Flow cytometry

Control or MEF2CA- lentivirus infected rosettes were kept in ultra low attachment plate for 2 weeks to form rosette spheres. These spheres were dissociated with accutase to make single cells for sorting by FACSDiVa (BD). Cells were collected into two separate tubes, GFP+ vs GFP-. Sorted cells were kept in suspension culture for 2 weeks to recover and expand under NPM. Spheres were subjected to the standard protocol for neural induction.

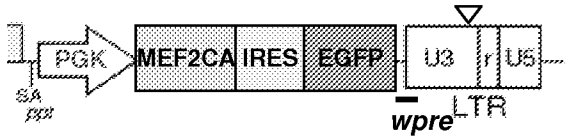
***In Vitro* Electrophysiology**

Sorted, GFP+ cells were plated onto 12- mm diameter glass cover slips coated with PO(100)/LN(10 μg/ml) (5x 10^4 cells per one well of 24-well plate) under TDM. 5 weeks later, whole-cell recordings were at performed at room temperature as previously described (Lei et al., 1992; Lipton et al., 1993; Kim et al., 1999; Choi et al., 2000). Cover slips were placed in a recording chamber with a volume of ~250 μl. The recording chamber was mounted on the stage of an Olympus IX71 inverted microscope. Electrical signals were amplified using an Axopatch 200B amplifier (Axon Instruments) and filtered at 5 kHz via a Bessel low pass filter. Data were sampled and analyzed using pClamp 10.1 software (Molecular Devices, USA). The patch pipettes were pulled from standard wall glass 1.5 mm in outer diameter (Warner) with final tip resistance of 5-12 MΩ. In general, for recording voltage-gated Na^+ currents, we used the following intracellular solution (in mM): CsCl 120, tetraethylammonium chloride (TEA-Cl) 20, HEPES 10, EGTA 2.25, $CaCl_2$ 1, $MgCl_2$ 2; pH adjusted to 7.4 with CsOH. To elicit

voltage-gated currents, we used 100 ms depolarizing steps from -60 to +80 mV in 20 mV increments following a 300 ms prepulse to -90 mV. For recording GABA evoked currents, the intracellular solution contained (in mM): 130 Cs-gluconate; 2 MgATP, 1 $MgCl_2$; 10 EGTA; 10 HEPES; pH adjusted to 7,25 with CsOH. Osmolarity was adjusted to 300 mOsm with sucrose. The bath solution contained a saline based upon Hanks' balanced salt solution (in mM): 137 NaCl, 1 $NaHCO_3$, 0.34 Na_2HPO_4, 2.5 KCl, 0.44 KH_2PO_4, 2.5 $CaCl_2$, 5 HEPES, 22.2 glucose; pH adjusted to 7.3 with NaOH. Receptor agonists and antagonists were prepared in bath solution and applied by an array of tubes placed 75-100 μm from the cells. Drug administration was controlled by a series of rapidly-triggered valves (The Lee Company and Warner Instruments, USA). Solution changes were achieved within 50-100 ms, and a pipette containing bath solution was used to rapidly washout applied drugs.

A. Lentiviral vector

- Lentiviral transfer vector
  : pRRLPGK/MEF2CA/IRES/GFPSIN18WPRE



- pCMVΔR8.74
- pMD.G plasmid

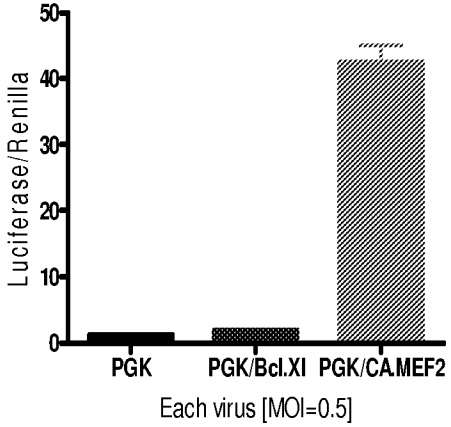
B. Titer

PGK.G : 1 x 10^9 IU/ml (control)

PGK.MEF2CA.IG : 1 x 10^8 IU/ml, (MEF2CA)

PGK.BclXl.IG : 4 x 10^8 IU/ml (Bcl.Xl)

C. MEF2C activity



D. Protein expression

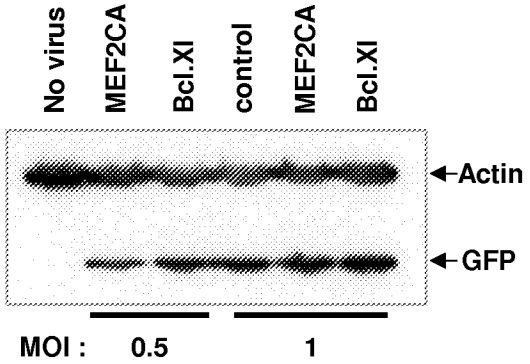


Fig.1 Biochemical charaterization of lenti-MEF2CA

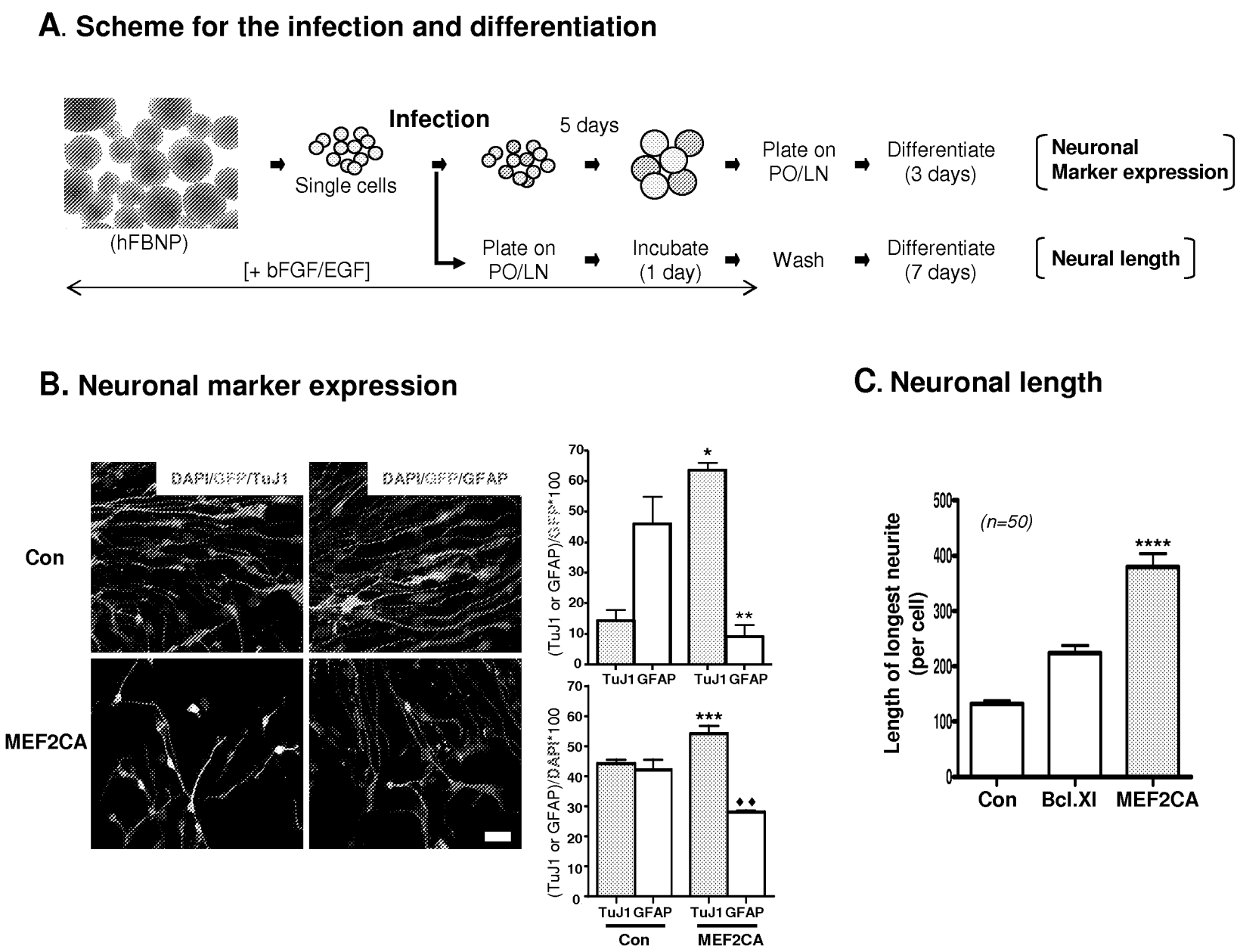


Fig.2 Effects of lenti-MEF2CA in human fetal brain-derived neural precursor cells

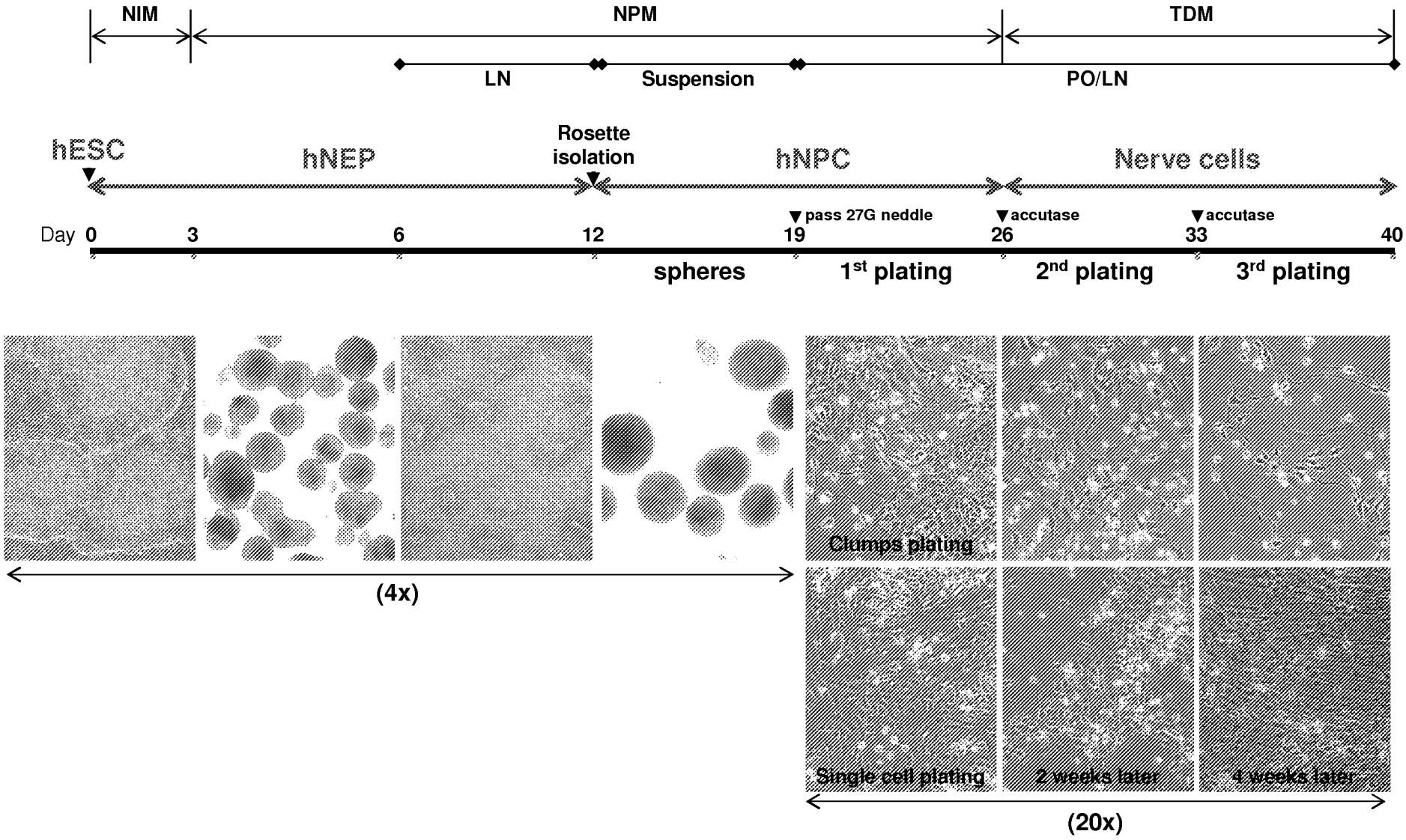


Fig.3 Scheme for the neural differentiation from human embryonic stem cells

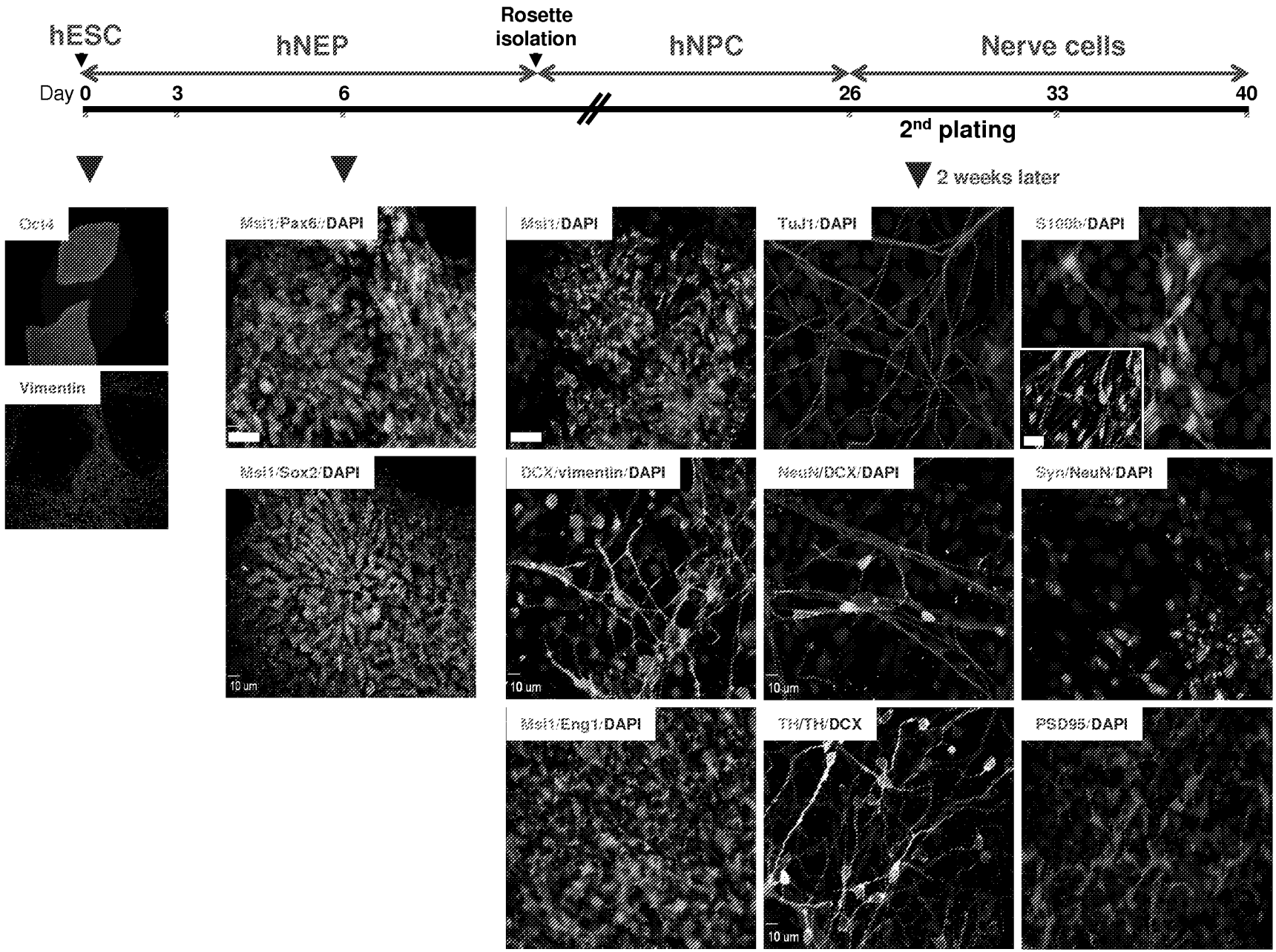


Fig.4 Marker protein expression during differentiation from hESC to neural cells

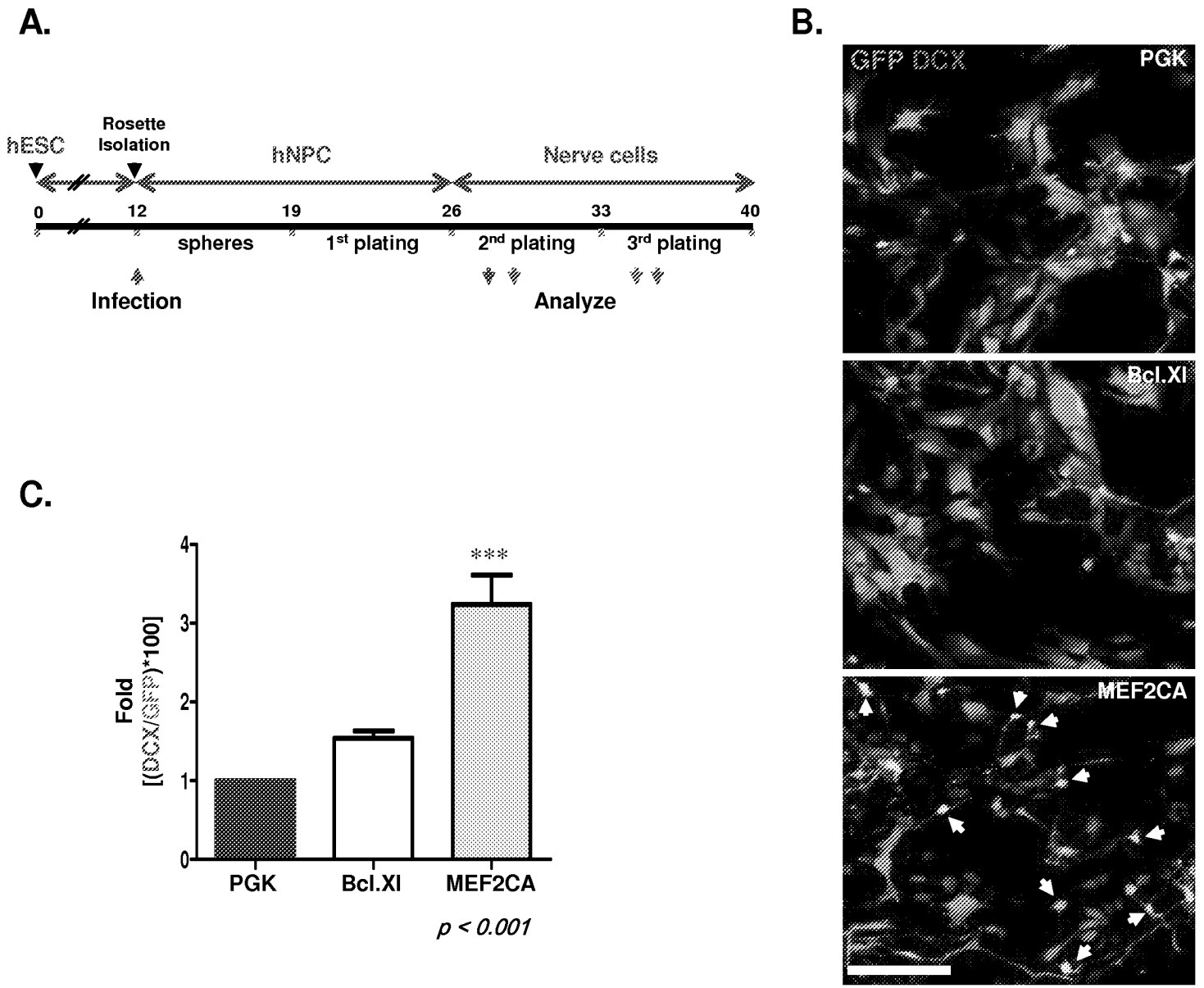


Fig.5 Enrichment of neuronal population in lenti-MEF2CA infected cells

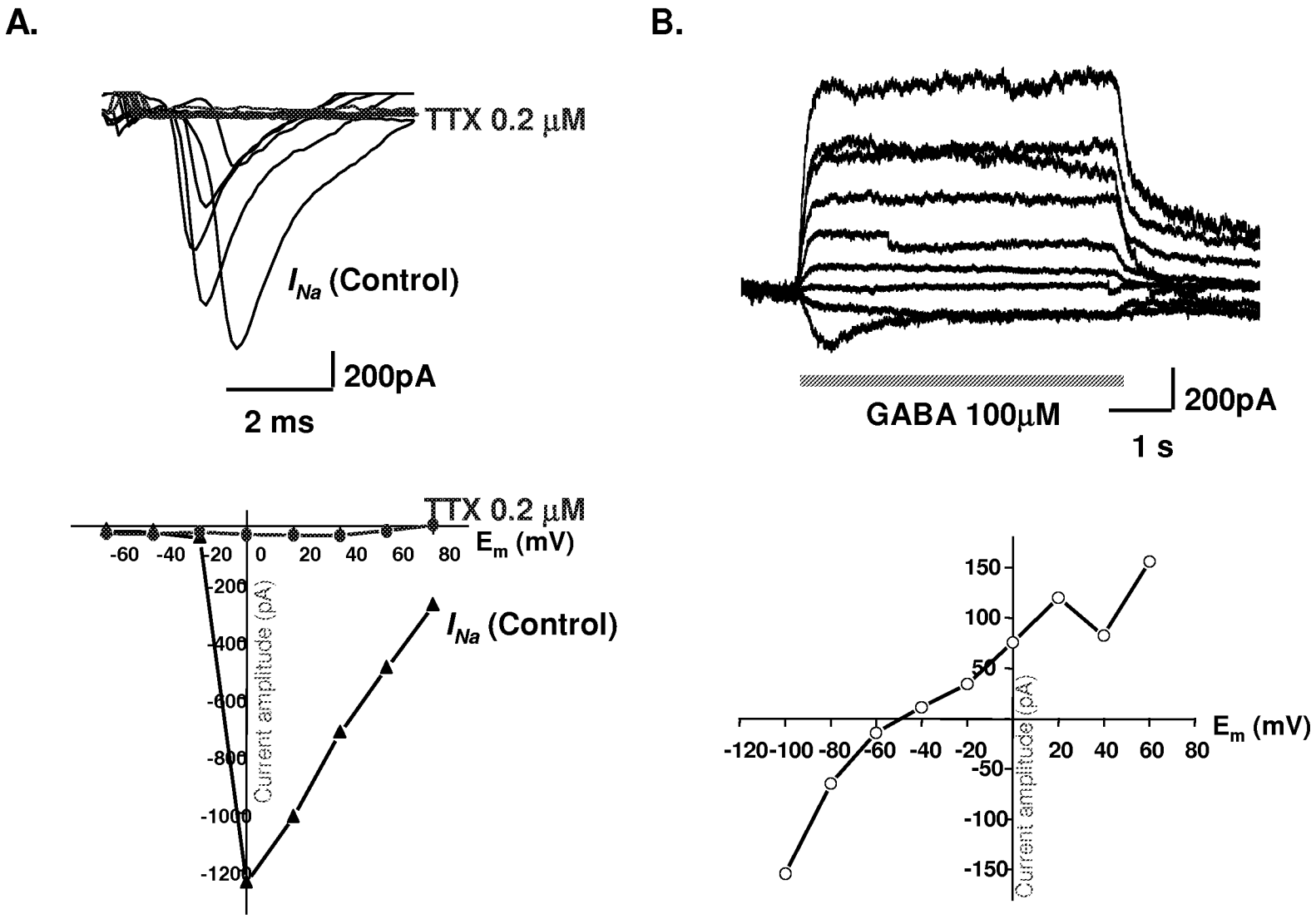


Fig.6 Electrophysiological activity of MEF2CA-derived neurons